APPLICATION UNDER UNITED STATES PATENT LAWS

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Invention: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS BY ATTENUATING THE sucC AND sucD GENES

Inventor (s): Bettina MÖCKEL
Walter PFEFFERLE
Achim MARX

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SPECIFICATION

New nucleotide sequences coding for the genes sucC and sucD

The present invention provides nucleotide sequences of coryneform bacteria coding for the genes sucC and sucD and a process for the fermentative production of amino acids, in particular L-lysine and L-glutamate, using bacteria in which the sucC- and/or sucD-gene is/are attenuated.

Prior Art

L-amino acids, in particular L-lysine and L-glutamate, are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry, and most particularly in animal nutrition.

It is known that amino acids can be produced by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum (C. glutamicum). On account of the great importance of amino acids efforts are constantly being made to improve the production processes. Improvements in production may involve fermentation technology measures, such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during fermentation or the working-up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods involving mutagenesis, selection and choice of mutants are used to improve the output properties. In this way strains are obtained that are resistant to antimetabolites or are auxotrophic for regulatorily important metabolites, and that produce amino acids.

For some years recombinant DNA technology methods have also been used to improve Corynebacterium strains producing L-amino acids.

Object of the Invention

The inventors have set themselves the task of providing new measures for improving the fermentative production of amino acids, in particular L-lysine and L-glutamate.

Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned hereinafter, it is understood that these terms refer to one or more amino acids, including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-lysine and L-glutamate are particularly preferred.

The present invention provides an isolated polynucleotide containing a polynucleotide sequence selected from the group comprising

a) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide, that contains the amino acid sequence of SEQ ID No. 2,

b) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide, that contains the amino acid sequence of SEQ ID No. 3,

c) polynucleotide coding for a polypeptide, that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

d) polynucleotide coding for a polypeptide, that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 3,

e) polynucleotide that is complementary to the polynucleotides of a), b), c) or d), and

f) polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b), c), d) or e),

the polypeptide preferably exhibiting the activity of succinyl-CoA synthetase.

The present invention also provides the polynucleotide according to claim 1, which is preferably a replicable DNA containing:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence that corresponds to the sequence (i) within the region of degeneration of the genetic code, or

(iii) at least one sequence that hybridizes with the sequence complementary to the sequence (i) or (ii), and optionally

(iv) functionally neutral sense mutations in (i).

The invention furthermore provides:

a polynucleotide according to claim 4, containing the nucleotide sequence as shown in SEQ ID No. 1,

a polynucleotide according to claim 1, wherein the polynucleotide is a preferably recombinant DNA replicable in coryneform bacteria,

a vector containing parts of the polynucleotide according to the invention, but at least 15 successive nucleotides of the claimed sequence,

and coryneform bacteria in which the sucC- and/or sucD-gene is/are attenuated in particular by an insertion or deletion.

The present invention moreover provides polynucleotides that substantially comprise a polynucleotide sequence, that can be obtained by screening a corresponding gene library by means of hybridization, that contains the complete sucC- and/or sucD-gene with the polynucleotide sequence corresponding to SEQ ID No. 1 with a probe that contains the sequence of the aforementioned polynucleotide according to SEQ ID No. 1 or a fragment thereof, and isolation of the aforementioned DNA sequence.

Polynucleotides that contain the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate cDNA, nucleic acids and/or polynucleotides or genes in their full length that code for succinyl-CoA synthetase, and to isolate such cDNA or genes whose sequence has a high similarity to that of the succinyl-CoA synthetase genes.

Polynucleotides that contain the sequences according to the invention are furthermore suitable as primers, by means of which DNA can be produced by the polymerase chain reaction (PCR) from genes that code for succinyl-CoA synthetase.

Such oligonucleotides serving as probes or primers contain at least 30, preferably at least 20, and most particularly preferably at least 15 successive nucleotides. Nucleotides with a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated from its natural environment.

"Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, in which connection these terms may refer to unmodified RNA or DNA or modified RNA or DNA.

By the term "polypeptides" are understood peptides or proteins that contain two or more amino acids bound via peptide bonds.

The polypeptides according to the invention include the polypeptides according to SEQ ID No. 2 and SEQ ID No. 3, in particular those having the biological activity of succinyl-CoA synthetase as well as those that are at least 70% identical to the polypeptide according to SEQ ID No. 2 or SEQ ID No. 3, and preferably at least 80% and particularly preferably at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 or SEQ ID No. 3 and that have the aforementioned activity.

The present invention furthermore relates to a process for the fermentative production of amino acids selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucin, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine, in particular L-lysine and L-glutamate, using coryneform bacteria that in particular already produce the amino acids, especially L-lysine and/or L-glutamate, and in which the nucleotide sequences coding for the sucC- and/or sucD-gene are attenuated, and in particular are expressed at a low level.

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that can be coded by the corresponding DNA, by for example using a weak promoter or a gene and/or allele that codes for a corresponding enzyme with a low activity and/or inactivates the corresponding gene and/or allele or enzyme (protein) and optionally combines these features.

The microorganisms that are the subject of the present invention can produce amino acids, in particular L-lysine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms may be types of coryneform bacteria, in particular of the genus Corynebacterium. In the genus

Corynebacterium there should in particular be mentioned the type Corynebacterium glutamicum, which is known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the type Corynebacterium glutamicum, are in particular the following known wild type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and mutants and/or strains obtained therefrom that produce L-amino acids, such as for example the L-lysine-producing strains:

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464 and
Corynebacterium glutamicum DSM 5714.

The new genes sucC and sucD coding for the enzyme succinyl-CoA synthetase (EC 6.2.1.5) have been isolated from C. glutamicum.

In order to isolate the sucC- and/or the sucD-gene or also other genes from C. glutamicum, a gene library of this microorganism is first of all cultivated in E. coli. The cultivation of gene libraries is described in generally known textbooks and handbooks. By way of example there may be mentioned the textbook by Winnacker: Gene und Klone,

Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A very well-known gene library is that of the E. coli K-12 strain W3110, which has been cultivated by Kohara et al. (Cell 50, 495 - 508 (1987)) in λ-vectors. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library from C. glutamicum ATCC13032 that has been cultivated with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326 (1992)) in turn describe a gene library obtained from C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)). O'Donohue (The Cloning and Molecular Analysis of Four Common Aromatic Amino Acid Biosynthetic Genes from Corynebacterium glutamicum. Ph.D. Thesis, National University of Ireland, Galway, 1997) describes the cloning of C. glutamicum genes using the λ Zap Expression system described by Short et al. (Nucleic Acids Research, 16: 7583).

In order to produce a gene library from C. glutamicum in E. coli, plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268) may also be used. Particularly suitable as hosts are those E. coli strains that are restriction-defective and recombinant-defective, such as for example the strain DH5α (Jeffrey H. Miller: "A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria", Cold Spring Harbour Laboratory Press, 1992).

The long DNA fragments cloned with the aid of cosmids or other λ-vectors may then in turn be sub-cloned into accessible vectors suitable for DNA sequencing.

Methods for DNA sequencing are described *inter alia* by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The DNA sequences that are obtained may then be investigated with known algorithms and/or sequence analysis programs, such as for example that of Staden (Nucleic Acids Research 14, 217-232(1986)), the GCG-program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)), the FASTA algorithm of Pearson and Lipman (Proceedings of the National Academy of Sciences USA 85,2444-2448 (1988)) or the BLAST algorithm of Altschul et al. (Nature Genetics 6, 119-129 (1994)) and compared with the sequence entries listed in publicly accessible data banks. Publicly accessible data banks for nucleotide sequences are for example those of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany) or those of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

The new DNA sequences of C. glutamicum coding for the sucC- and sucD-genes have been discovered, and as SEQ ID No. 1 are part of the present invention. The amino acid sequence of the corresponding proteins has furthermore been derived from the existing DNA sequences using the methods described above. The resultant amino acid sequences of the sucC- and sucD-gene product are shown in SEQ ID No. 2 and SEQ ID No. 3.

Coding DNA sequences that arise from SEQ ID No. 1 due to the degeneracy of the genetic code are also a subject of the invention. In the same way DNA sequences that hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a

subject of the invention. Finally, DNA sequences that are produced by the polymerase chain reaction (PCR) using primers obtained from SEQ ID No. 1 are also the subject of the invention.

The person skilled in the art will find information on identifying DNA sequences by means of hybridization in, *inter alia*, the handbook "The DIG System User's Guide for Filter Hybridization" published by Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The hybridization takes place under stringent conditions, in other words only hybrids are formed in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical. It is known that the thoroughness of the hybridization including the washing stages is influenced or even determined by varying the buffer composition, temperature and the salt concentration. The hybridization reaction is preferably carried out at a relatively low degree of thoroughness compared to the washing stages (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC-buffer for example may be used at a temperature of ca. 50 - 68°C for the hybridization reaction. In this connection probes may also be hybridized with polynucleotides that have less than 70% identity with the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This may be effected for example by reducing the salt concentration to 2x SSC and optionally subsequently to 0.5x SSC (The DIG System User´s Guide for Filter Hybridization, Boehringer Mannheim, Mannheim, Germany, 1995), a temperature of ca. 50 - 68°C being maintained. It is also optionally possible to reduce the salt concentration down to 0.1x SSC. By stepwise raising of the hybridization temperature in steps of ca. 1 - 2°C from 50 to 68°C,

polynucleotide fragments can be separated that exhibit for example at least 70% or at least 80% or at least 90% to 95% identity to the sequence of the probe that is used. Further instructions for hybridization are available on the market in the form of so-called kits (e.g. DIG Easy Hyb von der Firma Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1603558).

The person skilled in the art can find details of the enhancement of DNA sequences by means of the polymerase chain reaction (PCR) in, *inter alia*, the handbook by Gait: Oligonucleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has now been found that coryneform bacteria produce L-amino acids, in particular L-lysine, in an improved manner after attenuation of the sucC- and/or sucD-gene.

In order to achieve such an attenuation, either the expression of the sucC- and/or sucD-gene or the catalytic properties of the enzyme proteins can be reduced or switched off. Both measures may optionally be combined.

The reduction of the gene expression may be achieved by suitable culture conditions or by genetic alteration (mutation) of the signal structures of the gene expression. Signal structures of the gene expression are for example repressal genes, activator genes, operators, promoters, attenuators, ribosone bonding sites, the start codon and terminators. The person skilled in the art can find information on the above in for example patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Patek et al. (Microbiology 142: 1297 (1996)) and in known textbooks on genetics and molecular biology, such as for

example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or the textbook by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to an alteration and/or reduction of the catalytic properties of enzyme proteins are known in the prior art; there may be mentioned by way of example the work carried out by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", reports of the Jülichs Research Centre, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Overviews and summaries may be obtained from known textbooks on genetics and molecular biology, such as for example those by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Mutations cover such phenomena as transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations, as a result of which false amino acids are incorporated or the translation is prematurely arrested. Deletions of several codons typically lead to a complete suppression of the enzyme activity. Details of producing such mutations are part of the prior art and can be obtained from known textbooks on genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

A conventional method of mutating genes of C. glutamicum is the method of gene disruption and gene replacement described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the method of gene disruption a central part of the coding region of the gene that is of interest is cloned in a plasmid vector that can replicate in a host (typically E. coli), but not in C. glutamicum. Vectors that may be used include for example pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-Patent 5,487,993), pCR®Blunt (Firma Invitrogen, Groningen, Niederlande; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector that contains the central part of the coding region of the gene is then converted by conjugation or transformation into the desired strain of C. glutamicum.

The method of conjugation is described for example in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described for example in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a crossover event, the coding region of the affected gene is disrupted by the vector sequence and two incomplete alleles are obtained, each of which lacks the 3'- and the 5'-end. This method has been used for example by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) in order to switch off the recA-gene of C. glutamicum. The sucC- and/or sucD-gene may be switched off in this way.

In the method of gene replacement a mutation, such as for example a deletion, insertion or base exchange is produced *in vitro* in the gene that is of interest. The allele that is produced is in turn cloned in a vector that is not replicative for C. glutamicum and the vector is then converted by transformation or conjugation into the desired host for C. glutamicum. The incorporation of the mutation and/or of the allele in the target gene and/or in the target sequence is achieved after homologous recombination by means of a first crossover event effecting integration and an appropriate second crossover event effecting excision. This method has been used for example by Peters-Wendisch (Microbiology 144, 915 - 927 (1998)) in order to switch off the pyc-gene of C. glutamicum by means of a deletion. A deletion, insertion or a base exchange can be incorporated into the sucC- and/or sucD-gene in this way.

A deletion, insertion or a base exchange can be incorporated into the sucC- and/or sucD-gene in this way.

Furthermore, it was found that by means of one or more amino acid replacements in the sucC-protein (SEQ ID No. 2) selected from the group: replacement at position 22 by any other proteinogenic amino acid except L-proline, replacement at position 44 by any other proteinogenic amino acid except glycine, and replacement at position 170 by any other proteinogenic amino acid except L-alanine, an attenuation takes place and coryneform bacteria that carry the corresponding amino acid replacement produced amino acids in an improved way, in particular L-lysine and/or L-glutamic acid.

Particularly preferred are one or more amino acid replacements selected from the group: L-proline at position 22 by L-serine, glycine at position 44 by L-glutamic acid, and L-alanine at position 170 by L-threonine.

Most particularly preferred is an SucC-protein that contains L-serine at position 22, L-glutamic acid at position 44, and L-threonine at position 170, as shown in SEQ ID No. 5.

As shown in SEQ ID No. 4 the replacement of L-proline by L-threonine at position 22 of the amino acid sequence may preferably be effected by replacing the nucleobase cytosine at position 64 by thymine, the replacement of glycine by L-glutamic acid at position 44 of the amino acid sequence may preferably be effected by replacing the nucleobase guanine at position 131 by adenine, and the replacement of L-alanine by L-threonine at position 170 of the amino acid sequence may preferably be effected by replacing the nucleobase guanine at position 508 by adenine.

Conventional mutagenesis methods may be employed for the mutagenesis, using mutagenic agents such as for example N-methyl-N'-nitro-N-nitrosoguanidine or ultraviolet light.

Furthermore, *in vitro* methods may be used for the mutagenesis, such as for example a treatment with hydroxylamine (J. H. Miller: A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) or mutagenic oligonucleotides (T. A. Brown: Gentechnologie für Einsteiger, Spektrum Akademischer Verlag, Heidelberg, 1993) or the polymerase chain reaction (PCR), as described in the handbook by Newton and Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994).

The corresponding sucC alleles and genes are sequenced and incorporated into suitable hosts by for example the method of gene replacement.

The present invention accordingly also provides coryneform bacteria containing the SucC proteins, in which the amino acid sequence shown in SEQ ID No. 2 contain one or more replacements selected from the group: replacement at position 22 by any other proteinogenic amino acid except L-

proline, replacement at position 44 by any other proteinogenic amino acid except glycine, and replacement at position 170 by any other proteinogenic amino acid except L-alanine.

The invention accordingly furthermore provides polynucleotide sequences derived from coryneform bacteria that contain the genes or alleles coding for the aforementioned SucC proteins.

Furthermore it may be advantageous for the production of L-amino acids, in particular L-lysine, in addition to enhance, in particular to over-express, one or more enzymes of the relevant biosynthesis pathway, glycolysis, anaplerosis, citric acid cycle or amino acid export, in order to attenuate the sucC- and/or sucD-gene.

The expression "enhancement" describes in this connection increasing the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes or alleles, using a strong promoter or a gene or allele that codes with a high degree of activity for a corresponding enzyme (protein), and optionally combining these measure.

Thus, in the production of L-lysine and/or L-glutamate, in addition to the attenuation of the sucC- and/or sucD-gene, one or more of the genes selected from the following group may be enhanced, in particular over-expressed:

- the dapA-gene coding for dihydrodipicolinate-synthase (EP-B 0 197 335),

- the gap-gene coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

- the gene tpi coding for triosephosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene pgk coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc-gene coding for pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the mqo-gene coding for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the gene lysC coding for a feed-back resistant aspartate kinase (EP-B-0387527; EP-A-0699759; WO 00/63388)),
- the lysE-gene coding for the L-lysine-export (DE-A-195 48 222),
- the gene zwa1 coding for the Zwa1-protein (DE: 19959328.0, DSM 13115).

Moreover, it may be advantageous for the production of L-lysine and/or L-glutamate, in addition to the attenuation of the sucC- and/or sucD-gene, at the same time to attenuate, in particular to reduce the expression of one or more of the genes selected from the group comprising:

- the gene pck coding for phosphoenolpyruvate-carboxykinase (DE 199 50 409.1, DSM 13047),
- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478, DSM 12969),
- the gene poxB coding for pyruvate-oxidase (DE:1995 1975.7, DSM 13114),
- the gene zwa2 coding for the zwa2-protein (DE: 19959327.2, DSM 13113).

Furthermore it may be advantageous for the production of amino acid, in particular L-lysine and/or L-glutamate, in addition to the attenuation of the sucC- and/or sucD-gene to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms containing the polynucleotide according to claim 1 are also the subject of the invention and may be cultured continuously or batchwise in a batch process (batch cultivation) or in a fed batch or repeated fed batch process in order to produce L-amino acids, in particular L-lysine. An overview of known cultivation methods is given in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/ Wiesbaden, 1994)).

The culture medium to be used must suitably satisfy the demands of the relevant strains. Descriptions of culture media for various microorganisms are given in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

As carbon source there may be used sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as for example soya bean oil, sunflower oil, groundnut oil and coconut oil, fatty acids such as for example palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid. These substances may be used individually or as a mixture.

As nitrogen source there may be used organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or as a mixture.

As phosphorus source there may be used phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate, or the corresponding sodium-containing salts. The culture medium must furthermore contain salts of metals such as for Example magnesium sulfate or iron sulfate that are necessary for growth. Finally, essential growth substances such as amino acids and vitamins may, in addition to the substances mentioned above, be used. Apart from this, suitable precursors may be added to the culture medium. The aforementioned feedstock substances may be added to the culture in the form of a one-off addition, or may be metered in during the actual cultivation in a suitable way.

Alkaline compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water or acidic compounds such as phosphoric acid or sulfuric acid may be used in an appropriate manner in order to regulate the pH of the culture. Antifoaming agents such as for example fatty acid polyglycol esters may be used to prevent foam formation. Suitable selectively acting substances such as for example antibiotics may be added to the medium in order to maintain the stability of plasmids. Oxygen or oxygen-containing gas mixtures such as for example air are introduced into the culture in order to maintain aerobic conditions. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until a maximum yield of the desired product has been formed. This target is normally achieved within 10 hours to 160 hours.

A pure culture of the strain Escherichia coli DH5αmcr/pK18mobsacBsucDdel was filed according to the Budapest Convention on 29 September 2000 as DSM 13749 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany).

A pure culture of the strain Escherichia coli Top10/pCRBluntsucCint was filed according to the Budapest Convention on 29 September 2000 as DSM 13750 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany).

Methods for determining L-amino acids are known from the prior art. The analysis may be carried out as described for example by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography followed by ninhydrin derivatisation or may be carried out by reverse phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The present invention is described in more detail hereinafter with the aid of embodiments.

Example 1

Production of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences, USA 84:2160-2164), obtained from Stratagene (La Jolla, USA,

Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase. The cosmid-DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid-DNA treated in this way was mixed with the treated ATCC13032-DNA and the batch was treated with T4-DNA-ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of the Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217). In order to infect the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. Infection and titration of the cosmid bank were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells having been plated out on LB-agar (Lennox, 1955, Virology, 1:190) with 100 µg/ml ampicillin. Recombinant individual clones were selected after incubation overnight at 37°C.

Example 2

Isolation and sequencing of the genes sucC and sucD

The cosmid-DNA of an individual colony was isolated using the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) according to the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250).

After gel electrophoresis separation the cosmid fragments were isolated in the large region from 1500 to 2000 bp using the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1 obtained from Invitrogen (Groningen, Niederlande, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the frequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture having been incubated overnight with T4-ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was electroporated into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) and was plated out on LB-agar (Lennox, 1955, Virology, 1:190) with 50 µg/ml zeocin. The plasmid preparation of the recombinant clones was performed with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out according to the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academies of Sciences U.S.A., 74:5463-5467) as modified by Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The RR dRhodamin Terminator Cycle Sequencing Kit from PE Applied Biosystems(Product No. 403044, Weiterstadt, Germany) was used. The gel electrophoresis separation and analysis of the sequencing reaction was performed in a rotiphoresis NF acrylamide/bisacrylamide gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) together with the "ABI Prism 377" sequencing equipment from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data that were obtained were then processed using the Staden program package (1986, Nucleic

Acids Research, 14:217-231) Version 97-0. The individual sequences of the pZero1 derivates were assembled into a coherent Contig. The computer-assisted analysis of the coding region was performed with the program XNIP (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out with the BLAST search programs (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402), against the non-redundant data bank of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

The nucleotide sequence that was obtained is illustrated in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1206 base pairs, which was identified as sucC-gene, as well as an open reading frame of 882 base pairs, identified as sucD. The sucC-gene codes for a polypeptide of 402 amino acids, which is shown in SEQ ID No. 2. The sucD-gene codes for a polypeptide of 294 amino acids, which is shown in SEQ ID No. 3.

Example 3

3.1 Production of an integration vector for the integration mutagenesis of the sucC-gene

Chromosomal DNA was isolated from the strain ATCC 13032 according to the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). On the basis of the sequence of the sucC-gene for C. glutamicum known from Example 1 the following oligonucleotides were selected for the polymerase chain reaction (see SEQ ID No. 6 and SEQ ID No. 7):

primer sucC-in1:
5`CGC GCG AAT CGT TCG TAT 3`
primer sucC-in2:
5`CGC CAC CAA TGT CTA GGA 3`

The indicated primers were synthesised by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out

with the Pwo polymerase from Boehringer Mannheim (Germany, Product Description Pwo DNA Polymerase, Product No. 1 644 947) according to the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). With the aid of the polymerase chain reaction the primers permit the enhancement of an approximately 0.55 kb large internal fragment of the sucC-gene. The product enhanced in this way was checked by electrophoresis in a 0.8% agarose gel.

The enhanced DNA fragment was ligated into the vector pCR®Blunt II (Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) using the Zero Blunt™ Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K2700-20).

The E. coli strain TOP10 was then electroporated into the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach, Vol. I, IRL-Press, Oxford, Washington DC, USA, 1985). The selection of plasmid-carrying cells was performed by plating out the transformation batch onto LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) that had been supplemented with 25 mg/l of kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI followed by agarose gel electrophoresis (0,8%). The plasmid was named pCRBluntsucCint and is shown in Fig 1.

3.2 Deletion of the sucD-gene

For this purpose chromosomal DNA was isolated from the strain ATCC13032 by the method of Tauch et al. (1995, Plasmid 33:168-179). On the basis of the sequence of the sucD-gene for C. glutamicum known from Example 2 the oligonucleotides described hereinafter were selected for

producing the sucD deletion allele (see SEQ ID No. 8 to SEQ ID No. 11):

primer sucD-d1:
5′-CGA TGT GAT TGC GCT TGA TG -3′

deletion primer sucD-d2:
5′-ACC TCA CGC ATA AGC TTC GCA TGC TCT GAA CCT TCC GAA C -3′

deletion primer sucD-d3:
5′-GTT CGG AAG GTT CAG AGC ATG CGA AGC TTA TGC GTG AGG T -3′

primer sucD-d4:
5′-ATG AAG CCA GCG ACT GCA GA -3′

The relevant primers were synthesised by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out using the Pfu polymerase (Stratagene, Product. No. 600135, La Jolla, USA) and the PTC 100-Thermocyclers (MJ Research Inc., Waltham, USA). With the aid of the polymerase chain reaction the primers permit the enhancement of a sucD allele with internal deletion. The product enhanced in this way was tested by electrophoresis in a 0.8% agarose gel and was also sequenced as described by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

Example 4

4.1 Integration mutagenesis of the sucC-gene in the strain DSM 5715

The vector pCRBluntsucCint mentioned in Example 3.1 was electroporated into C. glutamicum DSM 5715 (EP 0 435 132) according to the electroporation method of Tauch et. al. (FEMS Microbiological Letters, 123:343-347 (1994)). The strain DSM 5715 is an AEC resistant L-lysine producer. The vector pCRBlunt-sucCint cannot independently replicate in

DSM5715 and accordingly only remains in the cellulose if it had integrated into the chromosome of DSM 5715. The selection of clones with pCRBluntsucCint integrated into the chromosome is performed by plating out the electroporation batch onto LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) that had been supplemented with 15 mg/l of kanamycin.

In order to detect the integration the sucCint fragment was labelled according to the method described in "The DIG System User's Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993) using the Dig-Hybridization Kit from Boehringer. Chromosomal DNA of a potential integrant was isolated according to the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and was cut in each case with the restriction enzyme SphI and HindIII. The resultant fragments were separated by means of agarose gel electrophoresis and hybridized at 68°C using the Dig-Hybridization Kit from Boehringer. The plasmid pCRBluntsucCint named in Example 3.1 had inserted itself into the chromosome of DSM5715 within the chromosomal sucC-gene. The strain was identified as DSM5715::pCRBluntsucCint.

4.2 Construction of the exchange vector pK18mobsacBsucDdel

The sucD-deletion derivative obtained in Example 3.2 was, after separation in an agarose gel (0.8%) using the Qiagenquick Gel Extraction Kit (Qiagen, Hilden, Germany), isolated from the agarose gel and then used with the mobilisable cloning vector pK18mobsacB (Schäfer et al. (1994), Gene 14: 69-73) for the ligation. This had previously been cleaved with the restriction enzymes XmaI- and XbaI, mixed with the sucD-deletion allele, and treated with T4-DNA-ligase (Amersham Pharmacia, Freiburg, Germany).

The E. coli strain DH5αmcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) was then electroporated with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach, Vol.1, ILR-Press, Cold Spring Harbor, New York, 1989). The plasmid-carrying cells were selected by plating out the transformation batch onto LB agar(Sambrock et al., Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor, New York, 1989) that had been supplemented with 25 mg/l of kanamycin.

Plasmid DNA was isolated from a transformant by means of the QIAprep Spin Miniprep Kit from Qiagen, and the cloned sucD-deletion allele was verified by means of sequencing by the company MWG Biotech (Ebersberg, Germany). The plasmid was named pK18mobsacBsucDdel. The strain was identified as E.coliDH5αmcr/pK18mobsacBsucDdel.

4.3 Deletion mutagenesis of the sucD-gene in the C. glutamicum strain DSM 5715

The vector pK18mobsacBsucDdel mentioned in Example 4.2 was electroporated according to the electroporation method of Tauch et al.,(1989 FEMS Microbiology Letters 123: 343-347). The vector cannot replicate independently in DSM 5715 and accordingly only remains in the cellulose if it has integrated into the chromosome. The selection of clones with integrated pK18mobsacBsucDdel was performed by plating out the electroporation batch onto LB-agar (Sambrock et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, New York, 1989) that had been supplemented with 15 mg/l of kanamycin. Cultivated clones were streaked out onto LB-agar plates containing 25 mg/l of kanamycin and incubated for 16 hours at 33°C.

In order to achieve the excision of the plasmid together with the complete chromosomal copy of the sucD-gene, the clones were then grown on LB-agar containing 10% sucrose. The plasmid pK18mobsacB contains a copy of the sacB-gene,

which converts sucrose into levansucrase that is not toxic for C. glutamicum. Accordingly only those clones in which the integrated pK18mobsacBsucDdel has in turn been excised can be grown on LB-agar containing sucrose. In the excision either the complete chromosomal copy of the sucD-gene or the incomplete copy together with the internal deletion can be excised together with the plasmid.

In order to detect whether the incomplete copy of sucD still remains in the chromosome, the plasmid pK18mobsacBsucDdel fragment was labelled according to the method described in "The DIG System User's Guide for Filter Hybridization" published by Boehringer Mannheim GmbH (Mannheim, Germany, 1993) using the Dig-Hybridization Kit from Boehringer. Chromosomal DNA of a potential deletion mutant was isolated according to the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) and was in each case cut into separate sections using the restriction enzymes SphI and PstI. The resultant fragments were separated by agarose gel electrophoresis and hybridized at 68°C using the Dig Hybridization Kit from Boehringer. On the basis of the resultant fragments it could be shown that the strain DSM5715 has lost its complete copy of the sucD-gene and instead only the deleted copy is still available.

The strain was identified as C. glutamicum DSM5715ΔsucD.

Example 5

5.1 Production of L-glutamate using the strain DSM 5715::pCRBluntsucCint

The C. glutamicum strain DSM5715::pCRBluntsucCint obtained in Example 4.1 was cultivated in a suitable nutrient medium for producing L-glutamate and the glutamate content in the culture supernatant was determined.

For this purpose the strain was first of all incubated for 24 hours at 33°C on agar plates with the corresponding

antibiotic (brain-heart agar with kanamycin (25 mg/l). A pre-culture was inoculated using this agar plate culture (10 ml of medium in a 100 ml Erlenmeyer flask). The full medium Cg III was used as medium for the pre-culture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast Extract	10 g/l
Glucose (separately autoclaved)	2% (w/v)

The pH was adjusted to pH 7.4

Kanamycin (25 mg/l) was added to this medium. The pre-culture was incubated on a shaker for 16 hours at 33°C at 240 rpm. A main culture was inoculated from this pre-culture so that the initial optical density (660 nm) of the main culture was 0.1 OD. The medium MM was used for the main culture.

Medium MM

CSL (Corn Steep Liquor)	5 g/l
MOPS (Morpholinopropanesulfonic acid)	20 g/l
Glucose (separately autoclaved)	50g/l
Salts:	
$(NH_4)_2SO_4$)	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4.7H_2O$	1.0 g/l
$CaCl_2.2H_2O$	10 mg/l
$FeSO_4.7H_2O$	10 mg/l
$MnSO_4.H_2O$	5.0 mg/l
Biotin (sterile filtered)	0.3 mg/l
Thiamine.HCl (sterile filtered)	0.2 mg/l
Fumarate (sterile filtered)	5.81 g/l
Leucine (sterile filtered)	0.1 g/l
$CaCO_3$	25 g/l

CSL, MOPS and the salt solution are adjusted with ammonia water to pH 7 and autoclaved. The sterile substrate and vitamin solutions as well as the dry autoclaved $CaCO_3$ are then added.

Cultivation takes place in a 10 ml volume in a 100 ml Erlenmeyer flask with baffles. Kanamycin (25 mg/l) was

added. Cultivation took place at 33°C and 80% atmospheric humidity.

After 24 hours the OD was measured at a measurement wavelength of 660 nm using the Biomek 1000 instrument (Beckmann Instruments GmbH, Munich). The amount of glutamate formed was measured in an amino acid analyser from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatisation with ninhydrin detection.

The result of the test is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-glutamate mg/l
DSM5715	10.4	20
DSM5715::pCRBlunt sucCint	3.9	154

5.2 Production of L-glutamate using the strain DSM5715ΔsucD

The C. glutamicum strain DSM5715/pK18mobsacBsucDdel obtained in Example 4.3 was cultivated in a nutrient medium suitable for producing L-glutamate and the glutamate content in the culture supernatant was measured.

For this purpose the strain was first of all incubated for 24 hours at 33°C on agar plates. A preculture was inoculated using this agar plate culture (10 ml medium in 100 ml Erlenmeyer flask). The full medium CgIII was used for the preculture. Kanamycin (25 mg/l) was added to this medium. The preculture was incubated on a shaker for 16 hours at 33°C and at 240 rpm. A main culture was

inoculated from this preculture so that the initial OD (660 nm) of the main culture was 0.1 OD. The medium MM was used for the main culture.

The cultivation was carried out in a 10 ml volume in a 100 ml Erlenmeyer flask equipped with baffles. Cultivation was carried out at 33°C and 80% atmospheric humidity.

After 72 hours the OD was measured at a measurement wavelength of 660 nm using a Biomek 1000 instrument (Beckmann Instruments GmbH, Munich). The amount of glutamate formed was measured with an amino acid analyser from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatisation with ninhydrin detection.

The result of the test is shown in Table 2.

Table 2

Strain	OD (660 nm)	L-glutamate mg/l
DSM5715	8.1	7
DSM5715ΔsucD	13.3	33

Brief Description of the Figures:

Fig. 1: Map of the plasmid pCRBluntsucCint.

Fig. 2: Map of the plasmid pK18mobsacBsucDdel

The acronyms and abbreviations used in Fig. 1 have the following meanings:

KmR:	Kanamycin resistance gene
Zeocin:	Zeocin resistance gene
HindIII	Cutting site of the restriction enzyme HindIII
SphI	Cutting site of the restriction enzyme SphI
EcoRI:	Cutting site of the restriction enzyme EcoRI
sucCint:	Internal fragment of the sucC-gene
ColE1 ori:	Replication origin of the plasmid ColE1

The acronyms and abbreviations used in Fig. 2 have the following meanings:

oriV:	ColE1-like origin of pMB1
sacB	The sacB-gene coding for the protein levansucrose
RP4mob:	RP4-mobilisation site
Kan:	Resistance gene for kanamycin
sucDdel':	5'-terminal fragment of the sucD-gene from C. glutamicum
sucDdel'':	3'-terminal fragment of the sucD-gene from C. glutamicum
SphI:	Cutting site of the restriction enzyme SphI
PstI:	Cutting site of the restriction enzyme PstI
XmaI:	Cutting site of the restriction enzyme XmaI
XbaI:	Cutting site of the restriction enzyme XbaI

SEQUENCE PROTOCOL

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<170> Patentee Ver. 2.1

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<223> sucD-gene

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ggctctaagc atgggccgga a atg gaa ttg gca gtg gat ctt ttt gaa tac 171
Met Glu Leu Ala Val Asp Leu Phe Glu Tyr
1 5 10

caa gca cgg gac ctc ttt gaa acc cat ggt gtg cca gtg ttg aag gga 219
Gln Ala Arg Asp Leu Phe Glu Thr His Gly Val Pro Val Leu Lys Gly
15 20 25

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45 50 55

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Lys Ala Gly Gly Val Arg Val Ala Pro Thr Ser Ala Gln Ala Phe Asp
60 65 70

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Ala Ala Asp Ala Ile Leu Gly Met Asp Ile Lys Gly His Thr Val Asn
75 80 85 90

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gtt gaa ggt ggc atg gag atc gag atc ctg gcg aag gaa aag cct gaa   555
Val Glu Gly Gly Met Glu Ile Glu Ile Leu Ala Lys Glu Lys Pro Glu
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gct ttg gca aag gtg gaa gtg gat ccc ctc act ggt att gat gag gac   603
Ala Leu Ala Lys Val Glu Val Asp Pro Leu Thr Gly Ile Asp Glu Asp
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aaa gcg cgg gag att gtc act gct gct ggc ttt gaa act gag gtg gca   651
Lys Ala Arg Glu Ile Val Thr Ala Ala Gly Phe Glu Thr Glu Val Ala
155                 160                 165                 170

gag aaa gtc att ccg gtg ctg atc aag atc tgg cag gtg tat tac gaa   699
Glu Lys Val Ile Pro Val Leu Ile Lys Ile Trp Gln Val Tyr Tyr Glu
                175                 180                 185

gag gaa gca aca ctc gtt gag gtg aac ccg ttg gtg ctc acg gat gac   747
Glu Glu Ala Thr Leu Val Glu Val Asn Pro Leu Val Leu Thr Asp Asp
            190                 195                 200

ggc gat gtg att gcg ctt gat ggc aag atc acg ctg gat gat aac gct   795
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gat ttc cgc cat gat aac cgt ggt gcg ttg gct gaa tct gcc ggt ggc   843
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cga atc ctc gcg gaa tat aac cac cct ttg gtc acc gtt gtg gag ggt   1275
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His Gly Gln Phe Ala Thr Ala Asn                             Met
395                 400

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420                 425                 430                 435

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                440                 445                 450

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Ile Asn Asp Thr Glu Leu Pro Val Phe Gly Thr Val Lys Glu Ala Met
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Leu Thr Tyr Gln Met Met Tyr Glu Leu Ser Asp Ile Gly Ile Ser Thr
    565                 570                 575

gcg att ggt att ggc ggt gac cca atc atc ggt aca acc cat atc gac   1950
Ala Ile Gly Ile Gly Gly Asp Pro Ile Ile Gly Thr Thr His Ile Asp
580                 585                 590                 595

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tct aag cac gtg aca aaa cca gtt gtg ggt tac gtg gca ggc ttt acc   2094
Ser Lys His Val Thr Lys Pro Val Val Gly Tyr Val Ala Gly Phe Thr
        630                 635                 640

gcc cct gaa gga aag acc atg ggg cat gct ggc gcc atc gtg aca ggt   2142
Ala Pro Glu Gly Lys Thr Met Gly His Ala Gly Ala Ile Val Thr Gly
    645                 650                 655

tca gaa ggc act gcg cga gca aag aag cat gca ttg gag gcc gtg ggt   2190
Ser Glu Gly Thr Ala Arg Ala Lys Lys His Ala Leu Glu Ala Val Gly
660                 665                 670                 675

gtt cgc gtg gga aca act ccg agt gaa acc gcg aag ctt atg cgt gag   2238
Val Arg Val Gly Thr Thr Pro Ser Glu Thr Ala Lys Leu Met Arg Glu
                680                 685                 690

gta gtt gca gct ttg taactaacag gccacagatc ttagctttga ccagcggatt   2293
Val Val Ala Ala Leu
            695

tgtggctaat cgcccggtct gtgtagagta ttcatctgtg cgcaggacag tgtgacaaac 2353

actgaatagt gcatggcttt aaggccctgt ggcgcagttg gttagcgcgc cgccctg    2410
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<210> 2
<211> 402
<212> PRT
<213> Corynebacterium glutamicum

<400> 2

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Met Glu Leu Ala Val Asp Leu Phe Glu Tyr Gln Ala Arg Asp Leu Phe
  1               5                  10                  15
Glu Thr His Gly Val Pro Val Leu Lys Gly Ile Val Ala Ser Thr Pro
             20                  25                  30
Glu Ala Ala Arg Lys Ala Ala Glu Glu Ile Gly Gly Leu Thr Val Val
         35                  40                  45
```

Lys Ala Gln Val Lys Val Gly Gly Arg Gly Lys Ala Gly Gly Val Arg
50 55 60
Val Ala Pro Thr Ser Ala Gln Ala Phe Asp Ala Ala Asp Ala Ile Leu
65 70 75 80
Gly Met Asp Ile Lys Gly His Thr Val Asn Gln Val Met Val Ala Gln
85 90 95
Gly Ala Asp Ile Ala Glu Glu Tyr Tyr Phe Ser Ile Leu Leu Asp Arg
100 105 110
Ala Asn Arg Ser Tyr Leu Ala Met Cys Ser Val Glu Gly Gly Met Glu
115 120 125
Ile Glu Ile Leu Ala Lys Glu Lys Pro Glu Ala Leu Ala Lys Val Glu
130 135 140
Val Asp Pro Leu Thr Gly Ile Asp Glu Asp Lys Ala Arg Glu Ile Val
145 150 155 160
Thr Ala Ala Gly Phe Glu Thr Glu Val Ala Glu Lys Val Ile Pro Val
165 170 175
Leu Ile Lys Ile Trp Gln Val Tyr Tyr Glu Glu Glu Ala Thr Leu Val
180 185 190
Glu Val Asn Pro Leu Val Leu Thr Asp Asp Gly Asp Val Ile Ala Leu
195 200 205
Asp Gly Lys Ile Thr Leu Asp Asp Asn Ala Asp Phe Arg His Asp Asn
210 215 220
Arg Gly Ala Leu Ala Glu Ser Ala Gly Gly Leu Asp Ile Leu Glu Leu
225 230 235 240
Lys Ala Lys Lys Asn Asp Leu Asn Tyr Val Lys Leu Asp Gly Ser Val
245 250 255
Gly Ile Ile Gly Asn Gly Ala Gly Leu Val Met Ser Thr Leu Asp Ile
260 265 270
Val Ala Ala Ala Gly Glu Arg His Gly Gly Gln Arg Pro Ala Asn Phe
275 280 285
Leu Asp Ile Gly Gly Gly Ala Ser Ala Glu Ser Met Ala Ala Gly Leu
290 295 300
Asp Val Ile Leu Gly Asp Ser Gln Val Arg Ser Val Phe Val Asn Val
305 310 315 320
Phe Gly Gly Ile Thr Ala Cys Asp Val Val Ala Lys Gly Ile Val Gly
325 330 335
Ala Leu Asp Val Leu Gly Asp Gln Ala Thr Lys Pro Leu Val Val Arg
340 345 350
Leu Asp Gly Asn Asn Val Val Glu Gly Arg Arg Ile Leu Ala Glu Tyr
355 360 365
Asn His Pro Leu Val Thr Val Val Glu Gly Met Asp Ala Ala Ala Asp
370 375 380
His Ala Ala His Leu Ala Asn Leu Ala Gln His Gly Gln Phe Ala Thr
385 390 395 400
Ala Asn

<210> 3
<211> 294
<212> PRT
<213> Corynebacterium glutamicum

<400> 3
Met Ser Ile Phe Leu Asn Ser Asp Ser Arg Ile Ile Ile Gln Gly Ile
1 5 10 15
Thr Gly Ser Glu Gly Ser Glu His Ala Arg Arg Ile Leu Ala Ser Gly
20 25 30
Ala Lys Leu Val Gly Gly Thr Asn Pro Arg Lys Ala Gly Gln Thr Ile
35 40 45

```
Leu Ile Asn Asp Thr Glu Leu Pro Val Phe Gly Thr Val Lys Glu Ala
     50                  55                  60
Met Glu Glu Thr Gly Ala Asp Val Thr Val Ile Phe Val Pro Pro Ala
 65                  70                  75                  80
Phe Ala Lys Ala Ala Ile Ile Glu Ala Ile Asp Ala His Ile Pro Leu
                 85                  90                  95
Cys Val Ile Ile Thr Glu Gly Ile Pro Val Arg Asp Ala Ser Glu Ala
            100                 105                 110
Trp Ala Tyr Ala Lys Lys Val Gly His Thr Arg Ile Ile Gly Pro Asn
        115                 120                 125
Cys Pro Gly Ile Ile Thr Pro Gly Glu Ser Leu Ala Gly Ile Thr Pro
    130                 135                 140
Ala Asn Ile Ala Gly Ser Gly Pro Ile Gly Leu Ile Ser Lys Ser Gly
145                 150                 155                 160
Thr Leu Thr Tyr Gln Met Met Tyr Glu Leu Ser Asp Ile Gly Ile Ser
                165                 170                 175
Thr Ala Ile Gly Ile Gly Gly Asp Pro Ile Ile Gly Thr Thr His Ile
            180                 185                 190
Asp Ala Leu Glu Ala Phe Glu Ala Asp Pro Glu Thr Lys Ala Ile Val
        195                 200                 205
Met Ile Gly Glu Ile Gly Gly Asp Ala Glu Glu Arg Ala Ala Asp Phe
    210                 215                 220
Ile Ser Lys His Val Thr Lys Pro Val Val Gly Tyr Val Ala Gly Phe
225                 230                 235                 240
Thr Ala Pro Glu Gly Lys Thr Met Gly His Ala Gly Ala Ile Val Thr
                245                 250                 255
Gly Ser Glu Gly Thr Ala Arg Ala Lys Lys His Ala Leu Glu Ala Val
            260                 265                 270
Gly Val Arg Val Gly Thr Thr Pro Ser Glu Thr Ala Lys Leu Met Arg
        275                 280                 285
Glu Val Val Ala Ala Leu
    290
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<210> 4
<211> 1206
<212> DNA
<213> Corynebacterium glutamicum

<220>
<221> CDS
<222> (1)..(1206)
<223> sucC-allele

<400> 4

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atg gaa ttg gca gtg gat ctt ttt gaa tac caa gca cgg gac ctc ttt   48
Met Glu Leu Ala Val Asp Leu Phe Glu Tyr Gln Ala Arg Asp Leu Phe
  1               5                  10                  15

gaa acc cat ggt gtg tca gtg ttg aag gga att gtg gca tca aca cca   96
Glu Thr His Gly Val Ser Val Leu Lys Gly Ile Val Ala Ser Thr Pro
             20                  25                  30

gag gcg gcg agg aaa gcg gct gag gaa atc ggc gaa ctg acc gtc gtc   144
Glu Ala Ala Arg Lys Ala Ala Glu Glu Ile Gly Glu Leu Thr Val Val
         35                  40                  45
```

```
aag gct cag gtc aag gtg ggc gga cgt ggc aag gcg ggt ggc gtc cgt   192
Lys Ala Gln Val Lys Val Gly Gly Arg Gly Lys Ala Gly Gly Val Arg
     50                  55                  60

gtg gca ccg acg tcg gct cag gct ttt gat gct gcg gat gcg att ctc   240
Val Ala Pro Thr Ser Ala Gln Ala Phe Asp Ala Ala Asp Ala Ile Leu
 65                  70                  75                  80

ggc atg gat atc aaa gga cac act gtt aat cag gtg atg gtg gcg cag   288
Gly Met Asp Ile Lys Gly His Thr Val Asn Gln Val Met Val Ala Gln
                85                  90                  95

ggc gct gac att gct gag gaa tac tat ttc tcc att ttg ttg gat cgc   336
Gly Ala Asp Ile Ala Glu Glu Tyr Tyr Phe Ser Ile Leu Leu Asp Arg
            100                 105                 110

gcg aat cgt tcg tat ctg gct atg tgc tct gtt gaa ggt ggc atg gag   384
Ala Asn Arg Ser Tyr Leu Ala Met Cys Ser Val Glu Gly Gly Met Glu
        115                 120                 125

atc gag atc ctg gcg aag gaa aag cct gaa gct ttg gca aag gtg gaa   432
Ile Glu Ile Leu Ala Lys Glu Lys Pro Glu Ala Leu Ala Lys Val Glu
    130                 135                 140

gtg gat ccc ctc act ggt att gat gag gac aaa gcg cgg gag att gtc   480
Val Asp Pro Leu Thr Gly Ile Asp Glu Asp Lys Ala Arg Glu Ile Val
145                 150                 155                 160

act gct gct ggc ttt gaa act gag gtg aca gag aaa gtc att ccg gtg   528
Thr Ala Ala Gly Phe Glu Thr Glu Val Thr Glu Lys Val Ile Pro Val
                165                 170                 175

ctg atc aag atc tgg cag gtg tat tac gaa gag gaa gca aca ctc gtt   576
Leu Ile Lys Ile Trp Gln Val Tyr Tyr Glu Glu Glu Ala Thr Leu Val
            180                 185                 190

gag gtg aac ccg ttg gtg ctc acg gat gac ggc gat gtg att gcg ctt   624
Glu Val Asn Pro Leu Val Leu Thr Asp Asp Gly Asp Val Ile Ala Leu
        195                 200                 205

gat ggc aag atc acg ctg gat gat aac gct gat ttc cgc cat gat aac   672
Asp Gly Lys Ile Thr Leu Asp Asp Asn Ala Asp Phe Arg His Asp Asn
    210                 215                 220

cgt ggt gcg ttg gct gaa tct gcc ggt ggc ttg gac att ttg gaa ctg   720
Arg Gly Ala Leu Ala Glu Ser Ala Gly Gly Leu Asp Ile Leu Glu Leu
225                 230                 235                 240

aag gcc aag aag aat gat ctg aac tac gtg aaa ctt gat ggc tct gtg   768
Lys Ala Lys Lys Asn Asp Leu Asn Tyr Val Lys Leu Asp Gly Ser Val
                245                 250                 255

ggc atc att ggc aat ggt gca ggt ttg gtg atg tcc acg ttg gat atc   816
Gly Ile Ile Gly Asn Gly Ala Gly Leu Val Met Ser Thr Leu Asp Ile
            260                 265                 270

gtg gct gca gct ggt gaa cgc cat ggt ggg cag cgc ccc gcg aac ttc   864
Val Ala Ala Ala Gly Glu Arg His Gly Gly Gln Arg Pro Ala Asn Phe
        275                 280                 285
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```
cta gac att ggt ggc gga gca tca gct gaa tcg atg gct gct ggt ctc   912
Leu Asp Ile Gly Gly Gly Ala Ser Ala Glu Ser Met Ala Ala Gly Leu
    290                 295                 300

gat gtg atc ctt ggg gat agc cag gta cgc agt gtg ttt gtg aat gtg   960
Asp Val Ile Leu Gly Asp Ser Gln Val Arg Ser Val Phe Val Asn Val
305                 310                 315                 320

ttt ggt ggc atc acc gcg tgt gat gtg gtg gca aag gga atc gtt gga  1008
Phe Gly Gly Ile Thr Ala Cys Asp Val Val Ala Lys Gly Ile Val Gly
                325                 330                 335

gct ttg gat gtg ctc ggc gat caa gca acg aag cct ctt gtg gtg cgc  1056
Ala Leu Asp Val Leu Gly Asp Gln Ala Thr Lys Pro Leu Val Val Arg
            340                 345                 350

ctt gat ggc aac aac gtg gtg gaa ggc aga cga atc ctc gcg gaa tat  1104
Leu Asp Gly Asn Asn Val Val Glu Gly Arg Arg Ile Leu Ala Glu Tyr
        355                 360                 365

aac cac cct ttg gtc acc gtt gtg gag ggt atg gat gca gcg gct gat  1152
Asn His Pro Leu Val Thr Val Val Glu Gly Met Asp Ala Ala Ala Asp
    370                 375                 380

cac gct gcc cat ttg gcc aat ctt gcc cag cac ggc cag ttc gca acc  1200
His Ala Ala His Leu Ala Asn Leu Ala Gln His Gly Gln Phe Ala Thr
385 .               390                 395                 400

gct aat                                                         1206
Ala Asn
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<210> 5
<211> 402
<212> PRT
<213> Corynebacterium glutamicum

<400> 5

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Met Glu Leu Ala Val Asp Leu Phe Glu Tyr Gln Ala Arg Asp Leu Phe
  1               5                  10                  15

Glu Thr His Gly Val Ser Val Leu Lys Gly Ile Val Ala Ser Thr Pro
             20                  25                  30

Glu Ala Ala Arg Lys Ala Ala Glu Glu Ile Gly Glu Leu Thr Val Val
         35                  40                  45

Lys Ala Gln Val Lys Val Gly Gly Arg Gly Lys Ala Gly Gly Val Arg
     50                  55                  60

Val Ala Pro Thr Ser Ala Gln Ala Phe Asp Ala Ala Asp Ala Ile Leu
 65                  70                  75                  80

Gly Met Asp Ile Lys Gly His Thr Val Asn Gln Val Met Val Ala Gln
                 85                  90                  95

Gly Ala Asp Ile Ala Glu Glu Tyr Tyr Phe Ser Ile Leu Leu Asp Arg
            100                 105                 110
```

Ala Asn Arg Ser Tyr Leu Ala Met Cys Ser Val Glu Gly Gly Met Glu
115 120 125

Ile Glu Ile Leu Ala Lys Glu Lys Pro Glu Ala Leu Ala Lys Val Glu
130 135 140

Val Asp Pro Leu Thr Gly Ile Asp Glu Asp Lys Ala Arg Glu Ile Val
145 150 155 160

Thr Ala Ala Gly Phe Glu Thr Glu Val Thr Glu Lys Val Ile Pro Val
165 170 175

Leu Ile Lys Ile Trp Gln Val Tyr Tyr Glu Glu Glu Ala Thr Leu Val
180 185 190

Glu Val Asn Pro Leu Val Leu Thr Asp Asp Gly Asp Val Ile Ala Leu
195 200 205

Asp Gly Lys Ile Thr Leu Asp Asp Asn Ala Asp Phe Arg His Asp Asn
210 215 220

Arg Gly Ala Leu Ala Glu Ser Ala Gly Gly Leu Asp Ile Leu Glu Leu
225 230 235 240

Lys Ala Lys Lys Asn Asp Leu Asn Tyr Val Lys Leu Asp Gly Ser Val
245 250 255

Gly Ile Ile Gly Asn Gly Ala Gly Leu Val Met Ser Thr Leu Asp Ile
260 265 270

Val Ala Ala Ala Gly Glu Arg His Gly Gly Gln Arg Pro Ala Asn Phe
275 280 285

Leu Asp Ile Gly Gly Gly Ala Ser Ala Glu Ser Met Ala Ala Gly Leu
290 295 300

Asp Val Ile Leu Gly Asp Ser Gln Val Arg Ser Val Phe Val Asn Val
305 310 315 320

Phe Gly Gly Ile Thr Ala Cys Asp Val Val Ala Lys Gly Ile Val Gly
325 330 335

Ala Leu Asp Val Leu Gly Asp Gln Ala Thr Lys Pro Leu Val Val Arg
340 345 350

Leu Asp Gly Asn Asn Val Val Glu Gly Arg Arg Ile Leu Ala Glu Tyr
355 360 365

Asn His Pro Leu Val Thr Val Val Glu Gly Met Asp Ala Ala Ala Asp
370 375 380

His Ala Ala His Leu Ala Asn Leu Ala Gln His Gly Gln Phe Ala Thr
385 390 395 400

Ala Asn

<210> 6
<211> 18
<212> DNA
<213> Corynebacterium glutamicum

<220>
<223> Primer sucC-in1

<400> 6
cgcgcgaatc gttcgtat 18

<210> 7
<211> 18
<212> DNA
<213> Corynebacterium glutamicum

<220>
<223> Primer sucC-in2

<400> 7
cgccaccaat gtctagga 18

<210> 8
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<223> Primer sucD-d1

<400> 8
cgatgtgatt gcgcttgatg 20

<210> 9
<211> 40
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Deletion primer

<220>
<223> Primer sucD-d2

<400> 9
acctcacgca taagcttcgc atgctctgaa ccttccgaac 40

<210> 10
<211> 40
<212> DNA
<213> Artificial sequence

<220>

<223> Description of the artificial sequence:
Deletion primer

<220>
<223> Primer sucD-d3

<400> 10
gttcggaagg ttcagagcat gcgaagctta tgcgtgaggt 40

<210> 11
<211> 20
<212> DNA
<213> Corynebacterium glutamicum

<220>
<223> Primer sucD-d4

<400> 11
atgaagccag cgactgcaga 20